PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: MODIFICATION OF A VIRUS TO REDIRECT INFECTIVITY AND ENHANCE TARGETED DELIVERY OF POLYNUCLEOTIDES TO CELLS

(57) Abstract

Targeted polynucleotide delivery to cells is enhanced by coupling to a virus capable of disrupting cellular endosomes, a molecular complex which redirects viral specificity to the targeted cell and carries the polynucleotide to be delivered. Viruses useful in this invention, such as adenovirus, are generally those which possess exposed capsid proteins and which are capable of disrupting endosomal vesicles upon internalization by a receptor-bearing cell. The modified virus can be used *in vivo, in vitro*, or *ex vivo* to redirect viral cell-binding specificity and to enhance the selective delivery of polynucleotides to target cells.

MODIFICATION OF A VIRUS TO REDIRECT INFECTIVITY AND ENHANCE TARGETED DELIVERY OF POLYNUCLEOTIDES TO CELLS

5   Background of the Invention

Delivery of polynucleotides to specific cells can be accomplished by exploiting the pathway of receptor-mediated endocytosis for cellular internalization. Carriers comprised of a cell-specific binding agent, such as a ligand for a cellular receptor (e.g., an asialoglycoprotein for the asialoglycoprotein receptor and transferrin for the transferrin

10   receptor), and a polynucleotide-binding agent, such as a polycation (e.g., polylysine) which can bind negatively-charged polynucleotides, can specifically target a polynucleotide to a receptor-bearing cell. See Wu, G.Y. and Wu, C.H. (1988) *J. Biol. Chem.* 263(29): 14261-14264; Wu, G.Y. and C.H. (1987) *J. Biol. Chem.* 262:4429-4432; and Zenke, M. et al. (1990) *Proc. Natl. Acad. Sci.* 87:3655-3659.

15   Several investigators have used the endosomolytic properties of adenovirus to enhance the introduction of agents into the internal compartments of cells. For example, FitzGerald et al. have shown that co-administration of adenovirus and polypeptides to cells in culture resulted in a marked increase in the delivery of those peptides into the cytosol of exposed cells ((1983) *Cell* 32:607-617). Others, such as Curiel et al. ((1992) *Am. J. Respir.*

20   *Cell Mol. Biol.* 6:247-252) and Cristiano et al. ((1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126) have shown that co-addition of adenovirus and protein-polylysine conjugates resulted in an increase in the efficiency of foreign gene expression when plasmid DNA was delivered to cells. The postulated mechanism behind this enhanced delivery of DNA is the ability of the virus to disrupt the endosome which contains delivered DNA. Normally, upon fusion

25   with lysosomes, the contents of these endosomes would be subject to degradative lysosomal enzymes. Instead, the cointernalized virus disrupts the endosome before it fuses with the lysosomal vesicles, thereby releasing the polynucleotide into the intracellular environment. While the virus-addition studies, cited above, showed enhancement of gene expression *in vitro*, the use of this technique *in vivo* is not likely to be practical because the non-targeted

30   virus cannot be efficiently directed to the same endocytotic vesicles as the complex. For this reason, forced co-localization by direct chemical linkage of the complex to the virus has been used by Wagner et al. who chemically coupled adenovirus to polylysine and formed transferrin-based ternary complexed with foreign DNA. This resulted in further enhancement of targeted gene expression (Wagner, E. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:6099-

35   6103). In these experiments, polylysine was coupled to adenovirus by random attachment of polycation to exposed glutamyl or lysyl residues of the virus. Curiel et al. demonstrated that in this system, both transferrin and adenoviral receptors were involved in the gene delivery ((1991) *Proc. Natl. Acad. Sci. USA* 88:8850-8854).

Summary of the Invention

This invention pertains to a modified virus having the capacity to disrupt cellular endosomal vesicles, useful for delivering a polynucleotide to a target cell for selective cellular internalization and to methods of preparing and using the modified virus. The

5   modified virus comprises a virus which has linked to its surface, a molecular complex comprised of a polynucleotide complexed with a carrier comprised of a cell-specific binding agent and a polynucleotide-binding agent. Coupling of the complex to the virus targets the virus to the target cell where it is internalized along with the polynucleotide through the same pathway. In this way, the capacity of the virus to disrupt endosomes is directly linked to the

10   polynucleotide being delivered to the cell. Coupling of the complex to the virus can also block the natural specificity of the virus and results in targeted gene expression directed by the attached cell-specific binding agent.

The virus can be any virus which is capable of disrupting endosomes upon internalization by a receptor-bearing cell. The polynucleotide delivered can be RNA or

15   DNA. The polynucleotides can be genes encoding a variety of proteins, including secretory proteins, cell surface proteins, and immunogenic proteins. In addition, the polynucleotide can be a ribozyme or an antisense construct which inhibits the expression of a specific gene or genes of cellular (e.g., a cellular oncogene) or of noncellular origin (e.g., a viral oncogene or the genes of an infecting pathogen such as a virus).

20   The cell-specific binding agent of the carrier is specific for a cellular surface structure, typically a receptor, which mediates internalization of bound ligands by endocytosis into cellular endosomes, such as the asialoglycoprotein receptor of hepatocytes. The cell-specific binding agent can be a natural or synthetic ligand (e.g., a protein, polypeptide, glycoprotein, carbohydrate, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a

25   cellular surface structure which then mediates internalization of the bound complex. The polynucleotide-binding agent of the carrier is a compound such as a polycation which stably complexes the polynucleotide under extracellular conditions and releases the polynucleotide under intracellular conditions in functional form.

The molecular complex can be introduced onto the surface of the virus by chemical

30   coupling either directly or through bridging agents. The modified virus can be used to deliver polynucleotides to cells *in vivo*, *in vitro*, or *ex vivo*.

Brief Description of the Drawings

Figure 1 (A-I) is photographs of cells following exposure to complexes of plasmid

35   DNA containing the gene for nuclear localizing β-galactosidase. Panels A-C, Huh7 [asialoglycoprotein (+)] cells; panels D-F, SK Hepl [asialoglycoprotein (-) cells; and panels G-I, HeLa S3 asialoglycoprotein (-)] cells. Cells exposed to AsOR-PL-DNA complex alone.

panels, A, D, and G; cells exposed to modified dl312 virus complexed to DNA, panels B, E, and H; cells treated with modified dl312 virus complexed to DNA plus a 1000-fold weight excess of AsOR (relative to AsOR content of the modified virus), panels C, F, and I).

5   **Detailed Description of the Invention**

A virus which disrupts cellular endosomes can be used to enhance expression of polynucleotides delivered to cells. According to this invention, a molecular complex which is targeted to a specific cell and which contains a polynucleotide to be delivered to the cell is coupled to the virus. Coupling of the molecular complex to the virus targets the virus for

10   cointernalization into the target cell along with the polynucleotide and through the same pathway. The coupling of the complex also results in alteration of viral infectivity by interfering with the virus' interaction with its natural receptor. Targeted expression of the polynucleotide is exclusively directed by the attached cell-specific binding agent. The cointernalized virus disrupts the endosomes containing the polynucleotide, thereby releasing

15   the polynucleotide into the cell and avoiding lysosomal degradation to result in increased expression of the polynucleotide by the cell.

Viruses useful in this invention are those which are capable of disrupting endosomal vesicles. Generally, these are viruses with exposed capsid proteins (nonenveloped viruses). However, some enveloped viruses, such as influenza virus, may be used. A preferred virus is

20   adenovirus. Particularly preferred is Type 5 adenovirus, available from American Type Culture Collection, which is noncarcinogenic. The virus can be replication defective or otherwise defective or truncated in structure or function as long as it retains or contains the component(s) necessary to disrupt endosomes (e.g., inactivated intact adenovirus).

The polynucleotide can be RNA or DNA. For example, targeted polynucleotides can

25   be genes encoding secretory proteins (see U.S. Patent Application Serial No. 710,558, filed on June 5, 1991, and Serial No. 893,736, filed on June 5, 1992), such as clotting factors and other blood proteins; cell surface proteins (see U.S. Patent Application Serial No. 695,598, filed on May 3, 1991), such as cell surface receptors for low density lipoproteins, for growth factors, or for hormones; immunogenic proteins (seeU.S. Patent Application Serial No.

30   699,891, filed on May 14, 1991, and Serial Number 882,669, filed on May 14, 1992), such as viral proteins (e.g., hepatitis B surface antigen or HIV envelope proteins) or protein of other pathogens. In addition, the polynucleotide can be an antisense polynucleotide (U.S. Patent Application Serial Number 864,003, filed on April 3, 1992). In other cases, the polynucleotide can be an RNA molecule which has catalytic activity (e.g., a ribozyme).

35   To form a molecular complex, a polynucleotide is complexed with a carrier comprised of a cell-specific binding agent and a polynocleotide-binding agent. The cell-specific binding agent is a molecule which specifically binds a cellular surface structure

which mediates its internalization by, for example, the process of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid, or a combination thereof. It is typically a surface receptor which mediates endocytosis of the ligand. Thus, the cell-specific binding agent can be a natural or synthetic ligand which binds the receptor. The ligand can

5   be a protein, polypeptide, glycoprotein or glycopeptide, carbohydrate, glycolipid, or a combination thereof which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be component of a biological organism such as a virus or a cell (e.g., mammalian, bacterial, protozoan).

The cell-specific ligand can also be an antibody, or an analogue of an antibody such

10   as a single chain antibody, which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, galactose-terminal carbohydrates such as carbohydrate trees obtained from natural glycoproteins, especially structures that either contain terminal galactose residues or can be enzymatically treated to expose terminal galactose residues, can

15   be used although other ligands such as polypeptide hormones may also be employed. In addition, naturally occurring plant carbohydrates such as arabinogalactan can be used as ligands. Other useful ligands for hepatocyte targeting include glycoproteins having exposed terminal carbohydrate groups such as asialoglycoproteins (galactose-terminal). These galactose-terminal ligands can be formed by coupling galactose-terminal carbohydrates such

20   as lactose or arabinogalactan to nongalactose-bearing proteins by reductive lactosamination. Examples of additional asialoglycoproteins include, but are not limited to, asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues.

25   For targeting the modified virus to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages, mannose-6-phosphate glycoproteins for fibroblasts, intrinsic factor-vitamin B12 for enterocytes and insulin for fat cells. Alternatively, the cell-specific ligand can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (e.g., an antigen which when bound is internalized) on the cell

30   surface. Such antibodies can be produced by standard procedures.

The polynucleotide-binding agent of the carrier complexes the polynucleotide to be delivered. Complexation with the polynucleotide must be sufficiently stable (either *in vivo* or *in vitro*) to prevent significant uncoupling of the polynucleotide extracellularly prior to internalization by the target cell. However, the complex must be cleavable under appropriate

35   conditions within the cell so the polynucleotide is released in functional form within the cell.

In a preferred embodiment, the binding between the polynucleotide-binding agent and the polynucleotide is based on electrostatic attraction which provides sufficient extracellular

stability, but is releasable intracellularly. Preferred polynucleotide-binding agents are
polycations that bind negatively charged polynucleotides. These positively charged proteins
can bind noncovalently with the polynucleotide to form a targetable molecular complex
which is stable extracellularly but releasable intracellularly. Suitable polycations are

5   polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines
and the like. A preferred polycation is polylysine. Other noncovalent bonds that can be used
to releasably link the expressible polynucleotide include hydrogen bonding, hydrophobic
bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide
antibodies bound to polynucleotide, and streptavidin or avidin binding to polynucleotide-

10   containing biotinylated nucleotides.

        The carrier of the molecular complex can be formed by chemically linking the cell-
specific binding agent to the polynucleotide-binding component. The chemical linkage is
typically covalent. A preferred linkage is a peptide bond. This can be formed with a water
soluble carbodiimide as described by Jung, G. et al. (1981) *Biochem. Biophys. Res. Commun.*

15   101: 599-606. Alternative linkages are disulfide bonds or strong noncovalent linkages as in
. avidin-biotin coupling.

        The chemical linkage can be optimized for the particular cell-specific binding agent
and polynucleotide-binding agent used to form the carrier. Reaction conditions can be
designed to maximize linkage formation but to minimize the formation of aggregates of the

20   carrier components. The optimal ratio of cell-specific binding agent to polynucleotide-
binding agent can be determined empirically. When polycations are used, the molar ratio of
the components will vary with the size of the polycation and the size of the polynucleotide.
In general, this ratio ranges from about 10:1 to 1:1, preferably about 5:1. Uncoupled
components and aggregates can be separated from the carrier by molecular sieve or ion

25   exchange chromatography (e.g., Aquapore^{TM} cation exchange, Rainin).

        The carrier is coupled to virus to form a modified virus. The coupling can be done
chemically. The chemical coupling is performed under conditions which preserve the virus'
ability to disrupt endosomes. The amount of carrier coupled to the virus is sufficient to target
the virus to the desired cell receptor and in certain embodiments, sufficient to inhibit the

30   specificity of the virus in unmodified form.

        In one embodiment, the coupling is performed by linking a carbohydrate on the viral
surface to amino groups of the carrier. An adenovirus, for example, has glycoprotein fibers
on its outer surface. The glycoprotein fibers contain the saccharide N-acetyl-glucosamine,
which is absent from other parts of the virus. The carrier can be covalently bound

35   specifically to these fibers through the N-acetyl-glucosamine. Viral N-acetyl-glucosamine is
oxidized to an aldehyde which reacts with the amino groups on, for example, the polylysine
component of the molecular complex to form a Schiff's base. The Schiff's base is stabilized

by reduction using a reducing agent such as sodium cyanoborohydride (NaCN(BH$_3$). Coupling the complex to the viral fibers redirects the virus to cells targeted by the receptor-specific ligand component of the complex and it abolishes the natural specificity of the adenovirus.

5   The polynucleotide can be complexed to the carrier by a stepwise dialysis procedure. In one embodiment, for use with carriers made of polycations such as polylysine, the dialysis procedure begins with a 2M NaCl dialyzate and ends with a 0.15M solution. The gradually decreasing NaCl concentrations results in binding of the polynucleotide to the carrier. In some instances, particularly when concentrations of the polynucleotide and carrier are low,

10   dialysis may not be necessary; the polynucleotide and carrier are simply mixed and incubated.

The molecular complex can contain more than one copy of the same polynucleotide or one or more different polynucleotides. The molar ratio of polynucleotide to the carrier can range from about 100(or more):1 to about 1:150 (or more), depending on the type and size of

15   carrier and polynucleotide.

The modified virus of this invention can be used to selectively deliver a polynucleotide(s) to a target cell under a variety of conditions. For *in vivo* delivery of a polynucleotide, the modified virus is administered to an organism in a physiologically acceptable vehicle. The modified virus can be administered parenterally. Preferably, it is

20   injected intravenously.

For *in vitro* delivery of a polynucleotide, cultured cells can be incubated with the modified virus in an appropriate medium under conditions conducive to endocytotic uptake by the cells. The modified viruses can also be used *ex vivo* to enhance polynucleotide delivery to cells or tissues which have been removed from an organism and will subsequently

25   be returned to that organism.

This invention is illustrated further by the following exemplification.

Exemplification

30   <u>Modification of Adenovirus by Coupling of an Asialoglycoprotein-Conjugate to the Carbohydrate Moiety of the Virus Alters Specificity of the Virus</u>

Materials and Methods

35   <u>Preparation of an Asialoorosomucoid-Poly L-lysine Conjugate</u>

A targetable conjugate (carrier) was prepared by mixing human plasma-derived asialoorosomucoid (AsOR) with poly L-lysine (PL), Mr = 41,000 (Sigma Chemical Co., St.

Louis, MO) in a 1: 1 weight ratio in 5 ml deionized water, and the solution adjusted to ph 7.4. The reactants were coupled by addition 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce Chemical Co., Rockville, IL) in a 140-fold molar excess over AsOR and stirred for 16 hrs at 25°C. The reaction mixture was then dialyzed against deionized water at 4°C for 72

5   hrs, lyophilized and purified by cation exchange chromatography using a high pressure liquid chromatographic system (Rainin Instrument Co., Woburn, MA) employing an Aquapore CX-300 1.0 cm X 25 cm cation exchange column (Rainin) with stepwise elution at a flow rate of 4.0 ml/min with 0.1 M sodium acetate, pH 5.0, 12 min; pH 2.5, 24 min; pH 2.25, 12 min; and pH 2.0, 14 min. The second peak eluted from the column, as detected by UV

10   absorption at 230 nm, was collected. This fraction was further purified by electrophoresis using an apparatus similar to the Bio-Rad Model 491 Prep Cell as described by that manufacturer, but with an acid-urea gel system as described previously by Panyim and Chalkley (*Arch. Biochem. Biophys.* (1969) 130:337-346). Bands were eluted with 0.35 M β-alanine-acetic acid, pH 4.8, and checked for UV absorption at 230 nm. The second peak

15   eluted was identified and confirmed as conjugate purified to homogeneity by an analytical acid-urea polyacrylamide gel electrophoresis. The purified conjugate was dialyzed against 0.025 M Tris-0.001 M EDTA (T-E) buffer through membranes with 12-14 kD exclusion limits.

20   <u>Propagation and Preparation of Adenovirus</u>

Type 5 adenovirus, kindly provided by Dr. Hamish Young, Columbia University, NY, was propagated and amplified in HeLa S3 cells as described previously (Green M. and M. Pina (1963) *Virology* 20: 909-207). To avoid potential cytotoxic effects of wild-type virus in studies on targeted gene expression, replication defective dl312 adenovirus were also

25   studied after propagation in 293 cells as described previously (Jones, N., and Schenk, T. (1979) *Proc. Natl. Acad. Sci USA* 76:3665-3669).

<u>Coupling of Adenovirus to AsOR-PL Conjugates</u>

Adenovirus samples, 1.0 X 10^12 particles, each in a total volume of 100μl 0.025 M

30   Tris-0.001 M EDTA (T-E) were reacted with 100 μl 0.02 M NaIO4 (Sigma) for 30 min at 25 °C in the dark. Then, 100 μl 0.20 M NaAsO$_2$ (Sigma), in T-E buffer was added for an additional 60 min. To quantitate the amount of conjugate coupled to adenovirus, AsOR-PL conjugate was radiolabeled with Na^125I by a chloramine-T method (Greenwood, F.C., et al. (1963) *Biochem. J.* 89:114-123) to a specific activity of 215 cpm/ng protein. Increasing

35   amounts of [^125I]-AsOR-PL from 20 μg to 1 mg (in terms of AsOR content) in 140 μl T-E buffer and 100μl 0.02 M NaBH3CN in T-E buffer were added to adenoviral samples and incubated for 15 hrs at 4°C. Samples were then applied on a discontinuous CsCl gradient

(Lawrence, W.C. and Ginsburg, H.S. (1967) *J. Virology* 1:851-867) (2.5 ml of CsCl at densities of 1.4, 1.3, 1.2 g/ml). Samples were ultracentrifuged at 25,000 rpm for 4 hrs at 4°C. Fractions, 350 μl each, were collected beginning at the top of the tubes, the radioactivity determined in each sample, and the fractions dialyzed against 150 mM NaCl, 10 mM EDTA,

5   10 mM Tris, pH 8.0. The radioactive peak found at the interface between 1.4 and 1.3 g/ml, where the virus usually bands (Lawrence, W.C., *supra*), was recovered and recentrifuged through another CsCl gradient to further purify the modified virus. Unbound conjugate remained at the top of the gradient tubes. Control samples containing a mixture of labeled conjugate, virus, and other reagents without periodate, failed to produce a radioactive band at

10   the density expected for the virus under identical conditions. For infectivity and gene transfection studies, virus was modified in an identical procedure as described above except that unlabeled virus was used in the coupling reaction.

       The number of modified viral particles in each fraction was determined by quantitation of DNA by UV absorption (Precious, B. and Russell, W.C. (1985) *A Practical*

15   *Approach In Virology*, Mahey, B.W.J., editor IRL Press/Oxford, England 193-205) after proteinase K-phenol-chloroform extraction (Rowe, D.W., et al. (1978) *Biochemistry* 17:1582-1590). In brief, purified modified virus was treated with SDS and proteinase K to make final concentrations of 0.1 and 100 μg/ml, respectively. The samples were incubated at 37°C for 1 hr followed by phenol-chloroform extraction, chloroform extraction and precipitation with

20   2.5 volumes of ethanol at -20°C overnight. The nucleic acid was collected by centrifugation at 10,000 rpm for 20 min at 4°C and the DNA concentration determined by measurement of the UV absorption at 260 nm. The maximum amount of AsOR-PL bound to virus was obtained with starting ratios of 20 μg of conjugate to 1×10^{11} viral particles. At this ratio, it was calculated that approximately 24 molecules of conjugate were bound to each viral

25   particle. Total protein content of the modified virus was determined by Bio-Rad assay as instructed by the manufacturer. Purified modified virus was filtered through 0.45μ membranes (Millipore) and remained stable in T-E buffer at 4°C for at least 2 weeks without loss of activity.

30   Formation of Complexed DNA and Modified Virus

       To form targetable complexes, plasmid DNA, 0.5 mg in 1 ml of 2 M NaCl was added in a ratio of 2 μg DNA to 0.4 μg modified virus, with respect to total protein. This ratio was determined optimal using an agarose gel retardation assay as described previously (Wu, G.Y. and Wu, C.H. (1987) *J. Biol. Chem.* 262:4429-4432). The sample was then placed in dialysis

35   tubing with an exclusion limit of 12-14 kD (Spectrapore), and step-wise dialyzed successively at 4°C for 0.5 hr against 1 l of NaCl in each of the following concentrations: 1.5

- 9 -

M, 1.0 M, 0.5 M, 0.25 M and 0.15 M. After the final dialysis, the complex was filtered through 0.45μ membranes prior to use in subsequent studies.

Cells and Cell Culture

5   To assess viral infectivity, three cell lines were used: human cervical carcinoma, HeLa S3 [asialoglycoprotein receptor (-)], human hepatoma, SK Hep1 [asialoglycoprotein receptor (-)] cells, and human hepatoma Huh 7 [asialoglycoprotein receptor (+)] cells (Liang T. J. et al J. (1993) *J. Clin. Invest.* 91: 1241-1246) were cultured in plastic dishes. Cells were seeded at densities of 5×10^5 cells in 35 mm plastic dishes containing minimal essential

10   medium (MEM) (GIBCO) and 5% fetal calf serum (GIBCO) under 5% CO_2 at 37°C.

Assays of Viral Infectivity

         Cells were allowed to attach for 24 hr after which time medium was removed and virus, modified or unmodified in medium, was added at concentrations of 100 viral

15   particles/cell. Virus samples were incubated with cells at 37°C for 48 hrs. Cells were then stained with trypan blue and counted microscopically. Infection assays were performed in triplicate and the results expressed as means ± S.E. of viable cells.

Assays for Targeted Gene Expression

20   To assay for gene transfection, a plasmid, pHBVsurf, containing the gene for human hepatitis B surface antigen (kindly provided by Dr. T. Jake Liang Massachusetts General Hospital, Boston, MA) (Liang, T.J. et al. (1993) *J. Clin. Invest.* 91:1241-1246) was used. The plasmid was grown in *E. coli*, isolated and purified as described previously (Birnboim, H.C. and Doly, J. (1979) *Nucleic Acis Res.* 7:1513-1518). Cells, seeded at $5.0 \times 10^5/35$ mm

25   dish, were treated 24 hrs later with 2 ml medium containing 21 μg DNA in the form of DNA complex alone, DNA complexed to modified wild-type virus, DNA complex plus wild-type virus, DNA complex plus modified dl312 virus, DNA complexed to modified dl312 virus, and DNA complexed to modified dl312 virus plus 1000-fold excess of AsOR by weight with respect to the AsOR content of the complex. In samples containing virus, approximately

30   2,000 viruses/cell were present and in competition studies a 1000-fold excess of viral particles/cell were used. All transfections were performed in triplicate and parallel dishes were prepared for cell counting. Medium, 200μl from each dish, was removed at 24 hrs and assayed for HBV surface antigen concentration by ELISA (Abbott) and expressed as pg/ 10^6 cells/24 hr ± S.E.

35   To determine the number of cells that express targeted genes, a plasmid pTZ βact nls lacZ Al, (a kind gift of Dr. Claire Bonnerot, Pasteur Institute, Paris, France) (Bonnerot. C. et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:6795-6799) containing the gene for β-galactosidase

- 10 -

with a nuclear localizing sequence was employed. Asialoglycoprotein receptor (+) and (-)
cells, all seeded at 5.0 X 10^5 cells/dish. were transfected separately, 24 hrs later, with DNA 2
µg in 2 ml medium as DNA complex alone. DNA complexed to modified dl312 adenovirus
at 2,000 particles/cell, DNA complexed to modified dl312 adenovirus plus a 1000-fold

5   excess of AsOR as described above. After 24 hrs of incubation, cells were fixed and washed
(Beddington, R.S.P. et al. (1989) *Development* 106:34-46), and then stained with X-gal
(Sanes, J.R. et al. (1986) *EMBO J.* 5:3133-3142) (Bethesda Research Lab, Gaithersburg,
MD) and the number of cells with blue-stained nuclei determined by counting
microscopically. The fraction of positive cells was calculated and expressed as means ± S.E.

10   of ten high power fields.

RESULTS

The effects of exposure of various preparations of adenovirus to HeLa S3, SK Hep1
and Huh 7 in terms of cell viability are shown in Table 1. Wild-type virus, as expected,

15   greatly decreased the number of viable cells of all three types. HeLa S3 cells were most
susceptible, with viable cells counts of only 10% at the conclusion of the incubation; Huh 7
were more resistant, having 31% viable, while SK Hep1 were intermediate at 14%, row 2.
However, row 3 shows that modification of wild-type virus by chemical coupling of
AsOR-PL conjugate resulted in a substantial reduction in the ability of virus to kill HeLa S3

20   and SK Hep1 cells, asialoglycoprotein receptor (-) cells; 91% and 86% of these cells remained
viable, respectively. In contrast, Huh7, asialoglycoprotein receptor (+) cells, remained
susceptible to modified virus; only 19% were viable under identical conditions. Row 4
shows that co-addition of a large excess of AsOR together with the modified virus effectively
blocked the infectivity of modified virus for Huh7 cells, but had no significant effect on the

25   infectivity towards the other cells. A similar competition had no effect on the infectivity of
wild-type virus alone, row 5. Exposure to neither the replication defective dl312, nor
modified dl312 virus resulted in any significant differences in the numbers of viable cells in
the three cell types compared to untreated controls.

- 11 -

Table 1

Effect of Modification of Adenovirus on Infection Specificity

Addition	HeLa S3*	% Control	Huh 7*	% Control	SK Hep1*	% Control
None	1.1 + .10	100	1.4 ± .13	100	1.4 ± .10	100
Wild-type	.11 ± .01	10	.43 ± .15	31	.20 ± .02	14
Modified wild-type	1.0 ± .05	91	.26 ± .02	19	1.2 ± .30	86
Modified wild-type + AsOR	1.0 ± .05	91	1.3 ± .02	93	1.4 ± .30	100
Wild-type + AsOR	.10 ± .05	9	.37 ± .03	27	.23 ± .20	16
dl312	1.2 ± .20	109	.98 ± .15	70	1.2 ± .11	86
Modified dl312	1.0 ± .11	91	1.3 ± .10	93	1.2 ± .11	86
Modified dl312 + AsOR	1.0 ± .10	91	1.4 ± .12	100	1.3 ± .12	93

* number of viable cells (x 10-6) determined by trypan blue exclusion.

The effect of modification of virus on foreign gene expression is shown in Table 2.
Complex alone produced 5.4 ± 1.1 pg HBV surface antigen/ 10^6 cells/24 hrs. DNA
complexed to modified wild-type, and dl312 virus resulted in expression 13- and 30-fold
greater than that of complex alone, respectively. Either wild-type or dl312 virus simply
added to AsOR-PL DNA complexes, but in the same proportions as in the modified viral
samples, resulted in only a 3-fold increase in expression compared to transfection with
complex alone. To examine the specificity of transfection by modified virus, a large excess
of AsOR was co-administered with complexed modified dl312 virus. This resulted in a
decrease in expression by more than 95%. However, co-administration of an excess of dl312
virus had no significant effect. This confirmed the infection specificity data, indicating that

- 12 -

the transfection by modified virus is mediated by recognition of the asialoglycoprotein
component and not the adenovirus portion of the complex.

Table 2

5

Effect of Modified Virus on Targeted Gene Expression

Addition	HBV surface antigen production (pg/10^6 cells/24hrs)
Untreated control cells	0
AsOR-PL-DNA complex alone	5.4 ± 1.1
Complex + wild-type virus	14 ± 2.5
Complex + dl312 virus	15 ± 5.5
Modified wild-type virus-DNA complex	70 ± 28
Modified dl312 virus-DNA complex	160 ± 15
Modified dl312 virus-DNA complex + 1000-fold AsOR	4.6 ± 1.0
Modified dl312 virus-DNA complex + dl312 virus	150 ± 25

10

15

20

Fig. 1 shows representative fields of cells following exposure to modified adenovirus
or controls complexed to plasmid DNA containing the gene for nuclear localizing β
-galactosidase. In Huh 7, asialoglycoprotein receptor (+) cells complex alone, panel A,
produced 0.5 ± 1%, but no positive cells in SK Hep 1 or HeLa S3, either receptor (-) cells,
25   panels D and G. respectively. However, modified dl312 virus complex exposed to Huh 7
cells produced 22 ± 2.1% positive cells, panel B, but none in SK Hep1 or HeLa S3, panels E
and F, repsectively. Finally, co-administration of a 1000-fold weight excess of AsOR to
compete with modified dl312 virus complex for asialoglycoprotein receptor uptake, panel C,
reduced the numbers of positive Huh 7 cells by more than 200-fold to 0.1 ± 0.5%. As with
30   modified dl312 complex alone, there were no positive cells in controls: SK Hep1 or HeLa S3
cells with AsOR competition. panels F and I, respectively.

In the above-described experiments, the contribution of the adenovirus receptors in
the cell entry process was eliminated in order to produce a transfection exclusively mediated
by the asialoglycoprotein ligand. Periodate oxidation, with its specificity for cleavage of
35   vicinal hydroxyl groups of the carbohydrates (Hughes, G. and Nevell, T.P. (1948) *Trans.
Faraday Soc.* 44:941-948), was selected in order to exclusively modify viral fibers. The
reaction results in the formation of aldehydes that can react with proteins to yield Schiff's

bases which, in turn, can be subsequently stabilized by reduction (Lane, C.F. (1975) *Synthesis* 3:135-146). Although it is known that the adenovirus fibers are required for viral attachment and internalization by target cells (Philipson, L. et al. (1968) *J. Virology* 2:1064-1075), the observed alteration in infection specificity as a result of our linkage procedure

5   could be due to steric or conformational changes of the fiber rather than a direct effect on the carbohydrate structure per se. Nevertheless, the infectivity and gene expression data support the conclusion that coupling of conjugates to adenovirus via a procedure that promotes carbohydrate-mediated linkages does result in alteration of viral infectivity, targeted gene expression exclusively directed by the attached ligand, and substantially increased foreign

10   gene expression.

Equivalents

Those skilled in the art will be able to recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described

15   herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

- 14 -

Claims

1.   A modified virus for delivering a polynucleotide to a cell, the virus being capable of disrupting endosomes and having a molecular complex linked to its surface, the molecular complex comprising a polynucleotide complexed with a carrier comprised of a cell-specific binding agent, which targets the virus to the cell, and a polynucleotide-binding agent.

2.   A modified virus of claim 1, in which cellular specificity of the virus in unmodified form has been substantially inhibited.

3   A modified virus of claim 1, wherein the virus is an adenovirus.

4.   A modified virus of claim 1, wherein the polynucleotide of the molecular complex is DNA.

5.   A modified virus of claim 1, wherein the polynucleotide of the molecular complex is RNA.

6.   A modified virus of claim 1, wherein the cell-specific binding agent of the carrier binds a surface receptor of the cell which mediates endocytosis of the virus by the cell.

7.   A modified virus of claim 6, wherein the cell-specific binding agent of the carrier is a ligand for an asialoglycoprotein receptor and the targeted cell bears an asialoglycoprotein receptor.

8.   A modified virus of claim 7, wherein the ligand of the carrier is an asialoglycoprotein.

9.   A modified virus of claim 7, wherein the ligand of the carrier is a galactose-terminal carbohydrate.

10.   A modified virus of claim 7, wherein the asialoglycoprotein receptor-bearing cell is an hepatocyte.

11.   A modified virus of claim 1, wherein the polynucleotide-binding agent of the carrier is a polycation.

12.   A modified virus of claim 11, wherein the polycation is polylysine.

13.   A modified virus of claim 1, wherein the cell-specific binding agent of the carrier is linked to the polynucleotide-binding agent by a covalent bond.

5   14.   A modified virus of claim 1, wherein the polynucleotide of the molecular complex is linked to the polynucleotide-binding agent of the carrier by a noncovalent bond.

15.   A modified virus of claim 1, wherein the molecular complex is linked to viral surface carbohydrates by a covalent bond.

10

16.   A modified adenovirus for delivering a polynucleotide to an asialoglycoprotein receptor-bearing cell, the adenovirus having a molecular complex linked to its glycoprotein surface fibers, the molecular complex comprising a polynucleotide complexed with a carrier comprised of a ligand for an asialoglycoprotein receptor and a polynucleotide-binding

15   polycation.

17.   A modified adenovirus of claim 16, wherein the ligand of the carrier is an asialoglycoprotein.

20   18.   A modified adenovirus of claim 16, wherein the ligand of the carrier is a galactose-terminal carbohydrate.

19.   A modified adenovirus of claim 16, wherein the polycation which binds the polynucleotide is polylysine.

25

20.   A modified adenovirus of claim 16, wherein the molecular complex is linked to the adenoviral glycoprotein surface fibers by a covalent bond.

21.   A method of delivering a polynucleotide to a specific cell of an organism, comprising

30   administering to the organism a virus having linked to its surface a molecular complex comprising a polynucleotide complexed with a carrier of a cell-specific binding agent and a polynucleotide-binding agent.

22.   A method of claim 21, wherein, in the administering step, the virus is an adenovirus.

35

23.   A method of claim 21, wherein, in the administering step, the polynucleotide of the molecular complex is DNA.

- 16 -

24.   A method of claim 21, wherein, in the administering step, the polynucleotide of the molecular complex is RNA.

5   25.   A method of claim 21, wherein, in the administering step, the cell-specific binding agent of the carrier binds a surface receptor of the cell which mediates endocytosis of the modified virus by the cell.

26.   A method of claim 21, wherein, in the administering step, the cell-specific binding
10   agent of the carrier is a ligand for an asialoglycoprotein receptor and the targeted cell bears an asialoglycoprotein receptor.

27.   A method of claim 26, wherein, in the administering step, the ligand of the carrier is an asialoglycoprotein.

15

28.   A method of claim 26, wherein, in the administering step, the ligand of the carrier is a galactose-terminal carbohydrate.

29.   A method of claim 26, wherein, in the administering step, the asialoglycoprotein
20   receptor-bearing cell is an hepatocyte.

30.   A method of claim 21, wherein, in the administering step, the polynucleotide-binding agent of the carrier is a polycation.

25   31.   A method of claim 30, wherein, in the administering step, the polycation is polylysine.

32.   A method of claim 21, wherein, in the administering step, the molecular complex is linked to viral surface carbohydrates by a covalent bond.

30

33.   A modified virus for delivering a polynucleotide to a cell, the virus being capable of disrupting endosomes in the cell, and having a carrier linked to it's surface, the carrier comprising a cell-specific binding agent which targets the virus to the cell and a polynucleotide-binding agent.

35

34.   A modified virus of claim 33, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

35.   A modified virus of claim 33, wherein the polynucleotide-binding agent is a polycation.
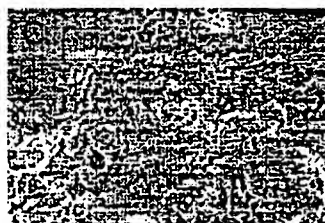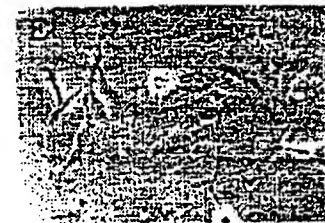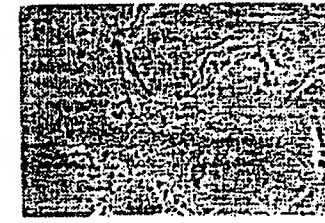
FIG. IA

FIG. IB

FIG. IC

FIG. ID

FIG. IE

FIG. IF

2/2

FIG. IG

FIG. IH

FIG. II

Intern .al Application No

PCT/US 93/09034

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   C12N15/87   A61K48/00   —

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   C12N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROC. NATL. ACAD. SCI. USA vol. 89 , 1992 pages 6099 - 6103 E. WAGNER ET AL. 'Coupling of adenovirus to transferrin-polylysine DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes' cited in the application	1,3-6, 11-14, 21-25, 30-33,35
Y	see the whole document	2,7-10, 16-20, 26-29,34
Y	WO,A,92 06180 (UNIVERSITY OF CONNECTICUT) 16 April 1992	2,7-10, 16-20, 26-29,34

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search

12 January 1994

Date of mailing of the international search report

14. 02. 94

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

SKELLY, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC NATL ACAD SCI USA vol. 89, no. 13 , 1992 pages 6094 - 6098 M. COTTEN ET AL. 'High-efficiency recepter-mediated delivery of small and large gene constructs ..' cited in the application see the whole document ---	1
A	PROC NATL ACAD SCI USA vol. 88, no. 19 , 1991 pages 8850 - 8854 D. CURIEL ET AL. 'Adenovirus enhancement of transferrin-polylysine mediated gene delivery' cited in the application see the whole document ---	1
A	BIOCONJUGATE CHEM. vol. 2, no. 4 , 1991 pages 226 - 231 E. WAGNER ET AAL. 'DNA-binding transferrin conjugates as functional gene delivery agents' see the whole document ---	1
X,P	WO,A,93 07283 (BOEHRINGER INGELHEIM INTERNATIONAL GMBH) 15 April 1993 see the whole document -----	1-35

2

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 21-32 are directed to a method of treatment of
(diagnostic method practised on) the human/animal body the search has been
carried out and based on the alleged effects of the compounds/compositions

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such
an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all
searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report
covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9206180	16-04-92	AU-A-	8860391	28-04-92
		CA-A-	2092323	02-04-92
		EP-A-	0553235	04-08-93
WO-A-9307283	15-04-93	AU-A-	2652692	03-05-93
		EP-A-	0545016	09-06-93